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13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information) To test our hypothesis that the HA binding peptide may be a new anti-neurofibromatosis agent via inducing apoptosis, we have proposed to focus on three aims: 1) To examine the anti-tumor effect of synthetic HA binding peptide on malignant neurofibromatosis cells; 2) To examine the anti-tumor effect of genetically expressed targeted HA binding peptide; 3) To examine the effect of targeted HA binding peptide on molecules involved in apoptosis. In the past first year, we have finished the following tasks: 1) chemical synthesis HA binding peptide and control peptide in a large scale; 2) identification of its HA binding activity; 3) characterization of anti-tumor activity of HA binding peptide; 4) study of the effect of HA binding peptide on molecules involved in cell programmed death; and 5) construction of mammalian expression vector for HA binding peptide. The results of study indicated that: 1) large scale synthesized HA binding peptide did possess HA binding activity; 2) synthetic HA binding peptide exerted an anti-tumor effect of on ST88-14 NF1 cells; 3) HA binding peptide could bind to Bcl-2/Bcl-x _L , the critical anti-apoptosis factors, which may be one of the mechanisms by which HA binding peptide inhibits ST88-14 NF1 cells; 4) the cells transfected with expression vector carrying cDNA of HA binding peptide could express this peptide as evidenced by Western blotting. In the next year, we will use the synthetic HA binding peptide and newly constructed expression vector to test if the <i>in vitro</i> anti-tumor effect of HA binding peptide can be translated <i>in vivo</i> against the cell growth of neurofibromatosis.				
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BODY

In neurofibromatosis, the loss or mutation of NF1 gene is the molecular error responsible for the phenotype. The defective neurofibromin leads to accumulation of hyperactive Ras-GTP, which make the mitogenic signals in NF1 cells "turn on" all the times, and the cells respond by proliferating. We speculated that HABP might be able to induce apoptosis and to reduce the immortality of NF1 cells.

For this, we are focusing on three aims: 1): To examine the anti-tumor effect of synthetic targeted HA binding peptide on malignant neurofibromatosis cells; 2): To examine the anti-tumor effect of genetically expressed targeted HA binding peptide; 3) : To examine the effect of targeted HA binding peptide on molecules involved in apoptosis.

In the past first year, we have finished the following tasks: 1) chemical synthesis of HA binding peptide and its control peptide in a large scale; 2) identification of HA binding activity of the synthetic peptides; 3) characterization of the anti-tumor activity of HA binding peptide; 4) study of the effect of HA binding peptide on molecules involved in cell programmed death; and 5) construction of mammalian expression vector for HA binding peptide.

The results are summarized as following:

1. Synthesis and characterization of HA binding peptide:

The HA binding peptide that we have proposed to test consists of 17 residues of amino acid: **KWCFRV CYRGIC YRRCR**, which consists of two domains of B[X7]B: **KWCFRV CYR** and **RGIC YRRCR**. Due to the four residues of cystine (C), this peptide forms two disulfide linkage, which exposes all six positively charged basic amino acids on its surface, allowing effective binding to its ligand or target (**Fig 1**). Furthermore, it confers stability to the peptide at low pH and high temperature, which makes it easy to manipulate and gives it a long shelf-life.

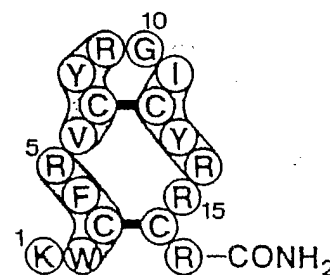


Fig 1. Structure of HA binding peptide

Due to its short sequence of amino acid, this HA binding peptide is relatively easy to make it by chemical synthesis. If HA binding peptide does exert a potent anti-tumor effect on neurofibromatosis, then practically it can be produced in a large quantity for the clinical trial.

The HA binding peptide and its scramble control peptide which has the same amount of amino acid were submitted to Genemed Synthesis Inc. (South San Francisco, CA) to synthesize chemically for the quantity of one gram. To avoid the enzyme degradation, the N-terminal were acetylated and the C-terminal amidated. The result of mass spectrophotometer analysis indicated that the synthetic peptide had molecular weight of 2309 Dalton with a relative purity (**Fig 2**).

To determine if this synthetic HA binding peptide has bioactivity, the binding assay was performed. The peptide was mixed with ^3H -HA, incubated for 2 hours and then applied to a nitrocellulose membrane using a dot blot apparatus. The free ^3H -HA was washed away with phosphate saline buffer, and then the membrane with retained peptide- ^3H -HA complex was cut out, emerged in scintillation fluid and the radioactivity was determined with a β -counter. The results (**Fig. 3**) showed that this synthetic targeted peptide had strong HA binding properties which could be blocked with a 50 fold excess of cold HA, while the scramble control peptide had very little background binding.

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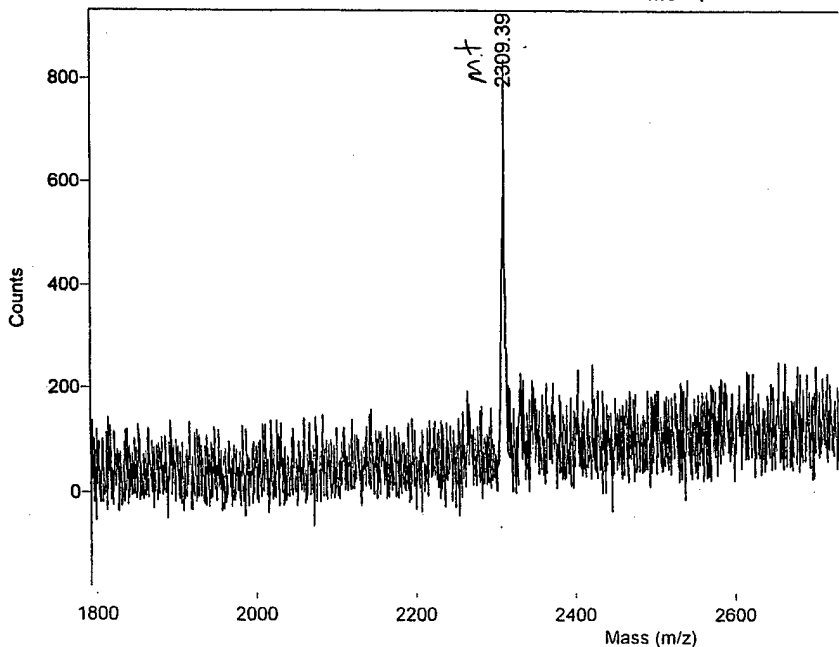


Fig 2. Mass spectrophotometer analysis of synthetic HA binding peptide.

Comment: Peptide-1 Lot #10011982

Method: PEPTIDE
Mode: Linear

Accelerating Voltage: 20000

Grid Voltage: 95.000 %

Guide Wire Voltage: 0.050 %

Delay: 50 ON

Laser: 2050

Scans Averaged: 54

Pressure: 5.04e

Low Mass Gate: OFF

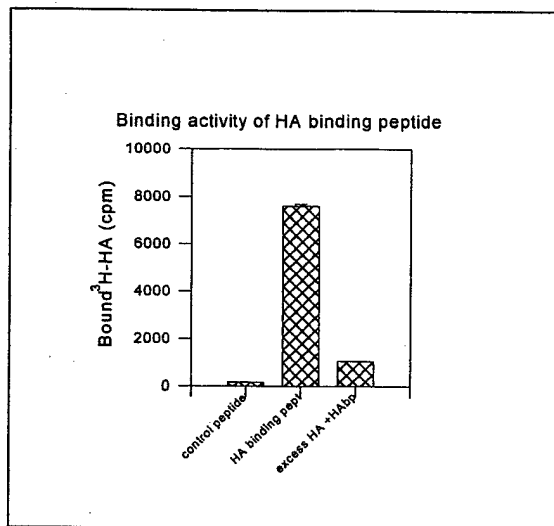


Fig 3. HA Binding activity of HA binding peptide. Fifty μg of targeted peptide was incubated with 20 μl of 100 $\mu\text{g}/\text{ml}$ of ^3H -HA (5×10^5 cpm/ μg HA) for 2 hours and loaded onto nitrocellulose membrane on a dot blot device. The free ^3H -HA was washed away with PBS, and then the membrane with retained peptide- ^3H -HA complex was cut out, emerged in scintillation fluid and the radioactivity was counted with a β -counter. The targeted peptide binds to HA.

The successful synthesis of HA binding peptide provided material for its anti-neurofibromatosis function study.

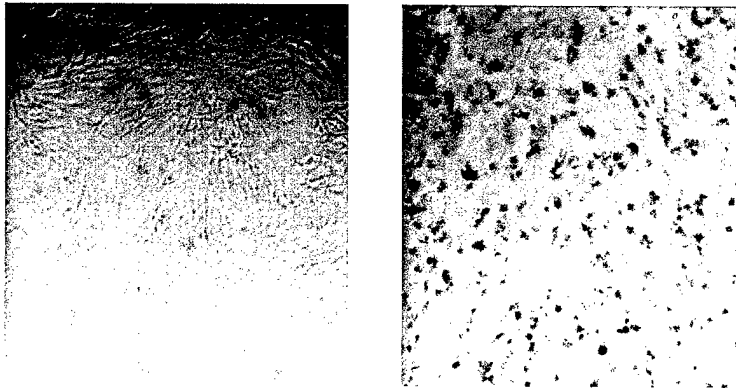
2. Effect of synthetic HA binding peptide on ST88-14 NF1 cells

Initially, we used HA binding peptide to treat the human ST88-14 NF1 cells and endothelial cells (human umbilical vein endothelial cells, HUVEC) in anchorage-dependent culture. It took less than 24 hours to cause a visible morphological change in HA binding peptide treated cells. The cells treated with HA binding peptide looked very sick, appearing rounded, condensed and detached (**Fig. 4 B and D**), much different from those treated with the control peptide (**Fig. 4 A and C**). HA binding peptide seems have

strong effect on cell viability. This effect of HA binding peptide on ST88-14 NF1 cells was compatible with Taxol, a most commonly used anti-tumor drug, which was used as positive control in the experiments (Fig. 5).

Fig. 4

Control peptide 100 $\mu\text{g/ml}$ HA binding peptide 100 $\mu\text{g/ml}$



Control peptide 200 $\mu\text{g/ml}$ HA binding peptide 200 $\mu\text{g/ml}$

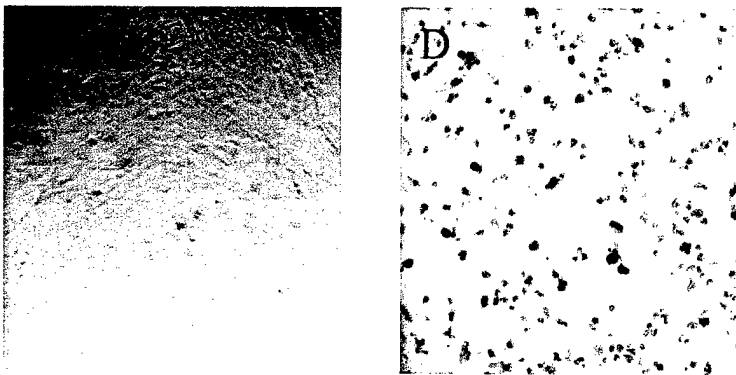


Fig. 5

Vehicle alone

A

Taxol 2 ng/ml

B

Taxol 10 ng/ml

C

Taxol 100 ng/ml



Fig 4 and 5. Effects of HA binding peptide and Taxol on ST88-14 cells. The cells were treated with indicated agents for 24 hours and then the pictures of altered morphology were taken under reversal microscopy.

To quantitatively measure the effect of HA binding peptide on cell proliferation, ^3H -thymidine (^3H -TdR) incorporation assay was carried out. The ST88-14 NF1 cells were cultured in 96 well plates and treated with different doses (25, 50, 100 or 200 $\mu\text{g/ml}$) of HA binding peptide for 24 hours, and then treated with ^3H -TdR to detect cell proliferation. The results showed that the HA binding peptide had a strong inhibitory effect on ST88-14 NF1 cells in a dose dependent manner ($P < 0.01$, Fig. 6). Furthermore, the proliferation of HUVEC induced by FGF2 was also inhibited by HA binding peptide ($P < 0.01$, Fig. 7). These inhibitory effects were very reproducible.

Importantly, when tested on non-tumorigenic, immobilized normal cell lines (Cos 7 and NIH 3T3), the inhibitory rate was much lower than that either on the ST88-14 cells or on the proliferating endothelial cells, indicating that HA binding peptide might be preferentially targeting tumor cells (data not shown).

Fig 6

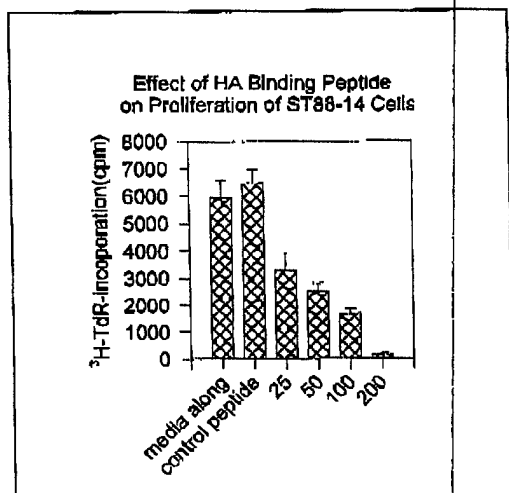


Fig 7

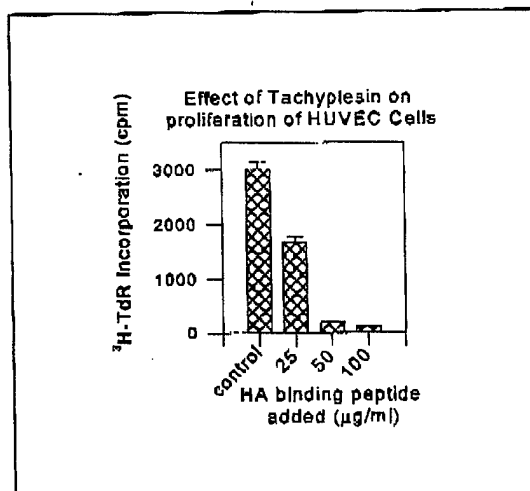


Fig 6 and 7. Effects of HA binding peptide on ST88-14 cells and HUVEC cells. The cells cultured in 96 well plate were treated with HA binding peptide at different doses for 24 hours and then traced with ³H-TdR for proliferation. The incorporated ³H-TdR was counted with β -counter.

These results indicated that the HA binding peptide did possess potent anti-tumor activity.

3. Molecular mechanisms for the action of HA binding peptide

To explore the underlying mechanism by which HA binding peptide exert its anti-tumor activity, we have carried out the studies focused on the molecules involved in cell apoptosis and proliferation. The results indicate that HA binding peptide could bind to anti-apoptosis molecules, such as Bcl-2/ Bcl-x_L. It could also reduce cyclin B1, Cdc-2 and phosphorylated erk. The data are showed as following:

A) Binding of HA binding peptide to Bcl-2

Bcl-2/ Bcl-x_L, the critical anti-apoptotic molecules, are anchored on membranes and may form a large macromolecular structure or lattice. This three-dimensional configuration is stabilized with the binding of Apaf-1, an adaptor for caspase 9. When a pro-apoptotic member of the Bcl-2 family binds to an anti-apoptotic member, the lattice conformation alters, forms ion-conducting channels [49-51], and releases Apaf-1 and cytochrome c from mitochondria into the cytosol. The entire lattice becomes conducive to caspase activation and apoptosis induction.

Obviously, the functional blockade of Bcl-2 / Bcl-x_L could restore the apoptotic process, and thereby, could inhibit the uncontrolled proliferation of ST88-14 NF1 cells.

To determine if HA binding peptide acts on Bcl-2/Bcl-x_L, we performed two types of experiments. First, an **ELISA-like binding assay** was performed. The HA binding peptide or control peptide were coated on maleic anhydride treated plates (special for coating of peptide, Micro membranes Inc.). After blocking, different concentrations of purified, human recombinant Bcl-2 protein (Sanda Cruz, Inc) were added to plate and incubated at room temperature for one hour. After wash, the anti-Bcl-2 was added for one hour followed

by peroxidase labeled second antibody and then substrate. While there was no binding in control peptide, the HA binding peptide bound strongly to Bcl-2 in a dose-dependent fashion (Fig. 8).

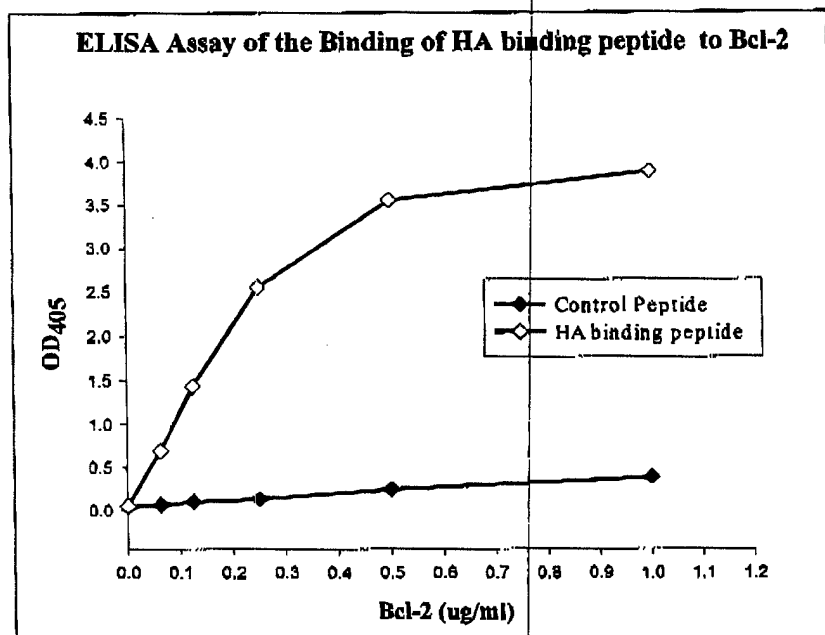
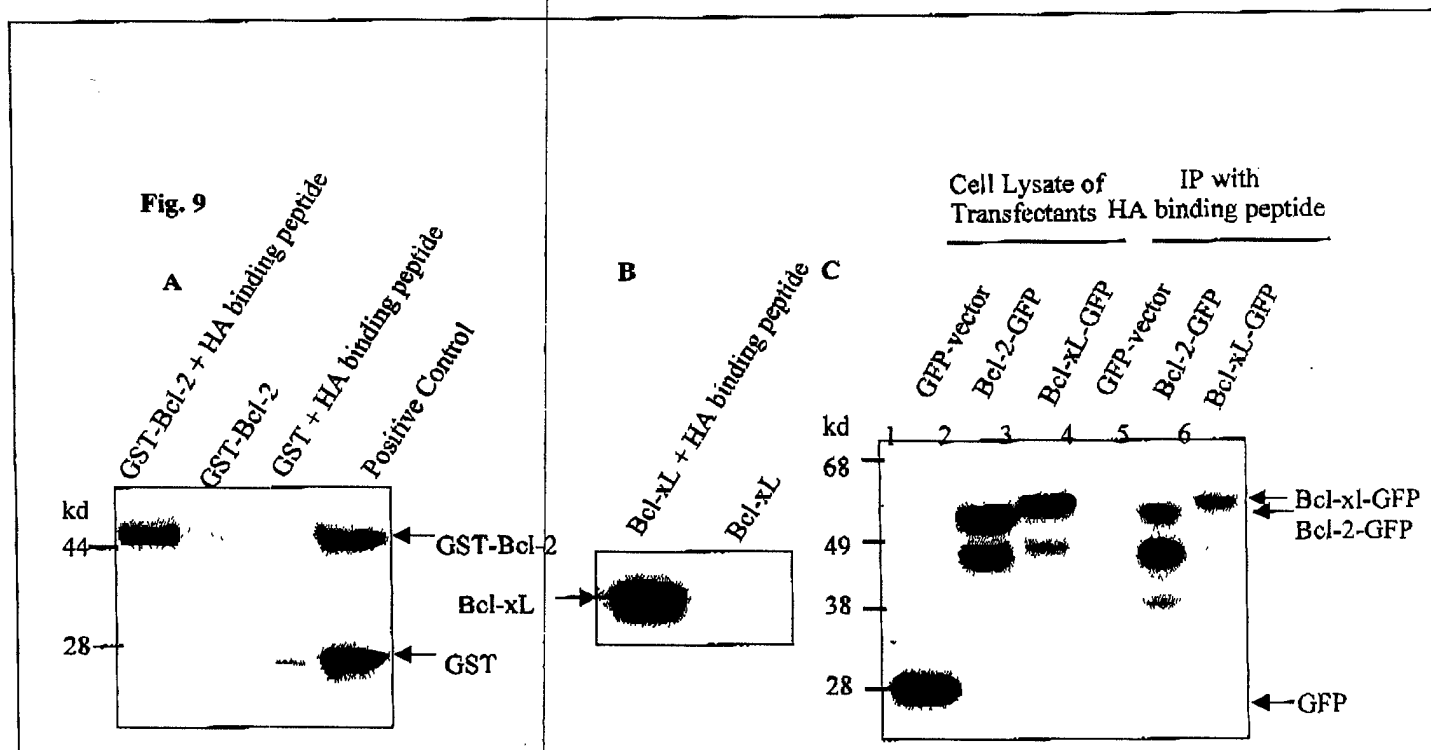


Fig. 8. Binding of HA binding peptide to Bcl-2. The High-Bond ELISA plates were coated with 200 µl of 5 µg/ml HA binding peptide or control peptide. After blocking each well with 5% BAS in TBS-T (10 mM Tris-Cl pH 7.4, 300 mM NaCl, 0.2% Tween-20), 100 µl of a series concentration of Bcl-2 (0 – 1.0 µg/ml) were added to each well of the plates and incubated at RT for one hour followed by incubation of anti-Bcl-2 antibody and then a second antibody-HRP. The plates are again washed with TBS-T and then each well was added 100 µl of a peroxidase substrate consisting 0.03% H₂O₂, 0.5 mg/ml 2,2' azinobis (3-ethylbenzthiazoline sulfonic acid) in 0.1M sodium citrate pH 4.2. After 30 min, the OD₄₀₅ will be determined using an ELISA reader.

To confirm if this is a true phenomenon and if HA binding peptide can also bind to Bcl-x_L, the immuno-precipitation and Western blotting was performed *in vitro*. 0.2 µg of purified GST-Bcl-2 fusion protein (Santa Cruz) or Bcl-x_L protein (expressed and purified from *E. coli*) or other GST fusion protein (as control for specificity) were incubated with (as test) or without (as control) 0.2 µg of biotinylated HA binding peptide in 500 µl of TBS-T buffer (300 mM NaCl, 1 mM EDTA, 20 mM Tris [pH 8.0], 0.2% Tween 20) at 4°C. One hour later, each of the binding reactions was mixed with 20 µl of 1:1 slurry of streptavidin-sepharose beads and incubated overnight at 4°C with gentle agitation. The beads were washed three times with 1 ml of TBS-T. The bound proteins were eluted in SDS-loading buffer, electrophoresized and transferred to nitrocellulose membrane followed by staining with anti-GST tag (for Bcl-2) or anti-Bcl-x_L secondary antibodies labeled with peroxidase and enhanced chemiluminescent (ECL) detection. The result (Fig. 9A) was very convincing: a major band was pulled down by streptavidin-sepharose beads in the mixture of GST-Bcl-2 and biotin-HA binding peptide (lane 1), indicating that the GST-Bcl-2 physically binds to HA binding peptide. This binding specificity was confirmed that no major band can be detected when **only** present of GST-Bcl-2 **without** biotin-HA binding peptide (lane 2) GST tagged **other protein** with biotin-HA binding peptide (lane 3). The majority of GST-Bcl-2 in the test tube was bound to biotin-HA binding peptide (lane 1) as compared to the loading control (lane 4). To our amazing, similar situation occurred in mixture of **Bcl-x_L with biotin-HA binding peptide** (Fig. 9B), in which there was a clear band in lane 1 while no band could be detected without addition of biotin-HA binding peptide (lane 2).

Does this happen *in vivo* in a natural condition? The 293T cells were transiently transfected pCMV vector carrying with Bcl-2 or Bcl-x_L cDNA fused at N-terminal of Green Fluorescence Protein (GFP) or with only GFP (as a control). Twelve hours later, cells were incubated with biotin-HA binding peptide for 3 hours and 1.0 ml of cell lysates were mixed with streptavidin-sepharose beads and incubated overnight at 4°C. The bound protein were eluted in SDS loading buffer analyzed in Western blotting with anti-GFP. The results showed that although all the cells (in lanes 4-5 of Fig. 9C) received biotin-HA binding peptide treatment, the streptavidin-sepharose beads could pulled down GFP fusion proteins only in Bcl-2-GFP or Bcl-x_L-GFP

transfected cells (Fig. 9C Lane 5, 6) not in vector alone transfectants (Fig. 9C Lane 4). The lysates of transfected cells (Fig. 9C Lanes 1-3) had multi-bands, indicating a degradation of expressed Bcl-2-GFP or Bcl-x_L-GFP fusion protein. We noticed that a truncated Bcl-2 (lower band in Fig. 9C, Lane 5) had a higher binding affinity to HA binding peptide than intact Bcl-2 (up band in Fig. 9C, Lane 5).



Figs. 9. The binding of HA binding peptide to Bcl-2 and Bcl-x_L: For *in vitro* binding assay, 0.2 µg of GST-Bcl-2 fusion protein or Bcl-x_L protein were incubated with or without 0.2 µg of biotin-HA binding peptide and incubated overnight with streptavidin-sepharose beads, followed by wash, elution and Western blotting with anti GST tag (for Bcl-2) or anti- Bcl-x_L and ECL detection (**A and B**). For *in vivo* co-immunoprecipitation, 293T cells in 100 mm dishes were transiently transfected pCMV vector with Bcl-2 or Bcl-x_L cDNA fused at N-terminal of GFP or with only GFP as a control. Twelve hours later, cells were incubated with biotin-HA binding peptide for 3 hours and harvested in 1.5 ml of lysis buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris [pH 8.0], 0.5% Nonidet P-40, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 1mM PMSF). One ml of cell lysates were mixed with streptavidin-sepharose beads and incubated overnight at 4°C. The beads were washed, eluted in 30 µl of SDS loading buffer. 10 µl of each transfected cell lysates and the immunoprecipitated sample were analyzed in Western blotting using anti-GFP antibody and ECL detection. (**C**).

These *in vitro* and *in vivo* data indicate that the inhibitory effect of HA binding peptide on ST88-14 NF1 cells may due to the binding of HA binding peptide to Bcl-2/Bcl-x_L, which blocks their anti-apoptosis function, leading to the cell programmed death.

4. Construction of mammalian expression vector for HA binding peptide

In this project, we proposed to use two approaches to test the anti- neurofibromatosis of HA binding peptide: one is to directly use the synthetic HA binding peptide, and the other is to use gene therapy. The is based on the rational that the molecular mass of HA binding peptide is so small that in the circulation it can be quickly filtered out by the kidney. Therefore, it is possible that the systemic delivery of peptide may not be able to reach the concentration necessary for the suppression of neurofibromatosis. However, this may be

overcame by a genetic engineering approach, which is one of the best ways to study the function of a given peptide.

The advantages of genetic engineering approach are as follows: 1) it results in a high concentration of peptides in the tumors; 2) the peptides should be evenly distributed since it should be expressed by almost all of the transfected tumor cells; and 3) consistent levels of peptides will be maintained by the tumors, since the transfected tumor cells have been genetically modified with cDNA coding for the designated peptide, and they will pass this property to their daughter cells. For these reasons, we believe that the approach of using expression vectors may give results not achieved by other deliver methods.

For the side-by-side comparison of the effects of two approaches, we need to have the peptide and the peptide expression vector available. We have obtained large quantity of HA binding peptide. We then constructed the expression vector for this peptide.

The amino acid sequence of HA binding peptide and control peptide will be back-translated into cDNA sequences. Two oligonucleotides that contain half of the cDNA code and some sequence overlap in the middle portion will be chemically synthesized, annealed and processed by PCR to obtain the complete sequence. In addition, restriction enzyme sites (*Kpn* I on 5'-end and *Not* I on 3'-end) will be placed at the ends of the cDNA for amplification by PCR. Following digestion, the PCR products will be inserted into the expression vector, p-IgK-HA (Fig. 10). This vector contains: 1) a CMV promoter; 2) a human Ig κ -chain leader sequence for secretion of the expressed peptides, and 3) a HA tag for detection of the expressed peptides in transfected cells. After the correct sequences have been confirmed by DNA sequencing, the HA vector for the expression of binding peptide was transiently transfected into 293 to see if the peptide is expressed. The cell lysate from the transfected 293 cells was subjected to Western blotting with anti-HA tag. The result (Fig 11) showed that there was one band on the Western blotting with a right molecular weight, indicating that the constructed vector is capable for expression of HA binding peptide.

Fig.10:

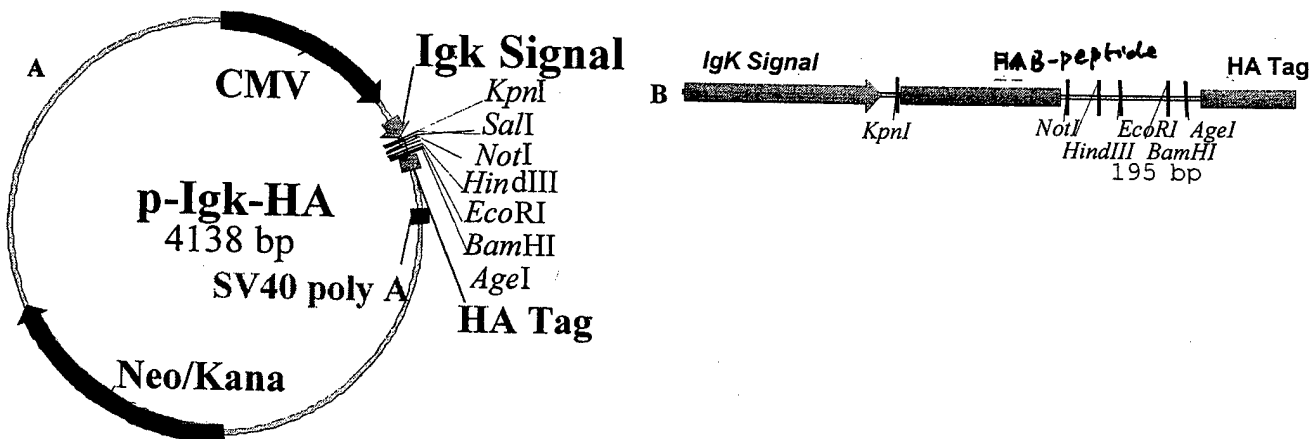


Fig. 10. Constructed vector for expression of HA binding peptide. Vector p-IgK-HA contains: a CMV promoter; a human Ig κ -chain leader sequence, cDNA for HA binding peptide and a HA tag.

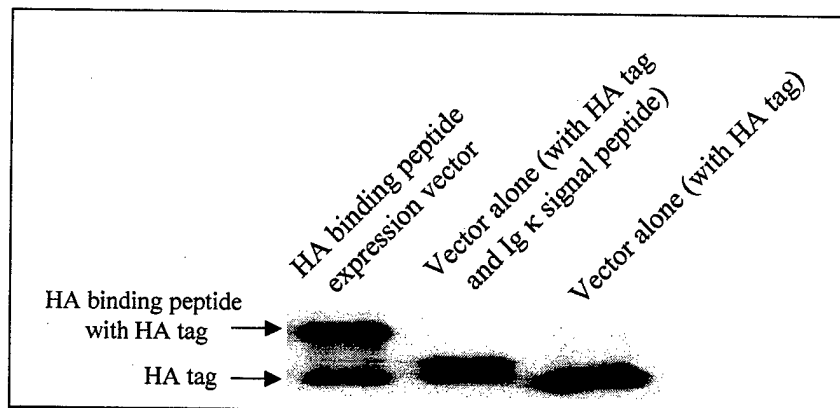


Fig. 11. The expression of HA binding peptide. Thirty μ g of transfected 293 cell lysate was subjected to Western blotting analysis with anti-HA. The band with right molecular weight was showed.

Thus far, we have successfully obtained both synthetic peptide and its expression vector, which provides us the necessary material for the *in vivo* test in next year.

In summary, in the past year, we have finished the following tasks: 1) chemical synthesis HA binding peptide and control peptide in a large scale; 2) identification of its HA binding activity; 3) characterization of anti-tumor activity of HA binding peptide; 4) study of the effect of HA binding peptide on molecules involved in cell programmed death; and 5) construction of mammalian expression vector for HA binding peptide.

The results of study indicated that: 1) large scale synthesized HA binding peptide did possess HA binding activity; 2) synthetic HA binding peptide exerted an anti-tumor effect of on ST88-14 NF1 cells; 3) HA binding peptide could bind to Bcl-2/Bcl-x_L, the critical anti-apoptosis factors, which may be one of the mechanisms by which HA binding peptide inhibits ST88-14 NF1 cells; 4) the cells transfected with expression vector carrying cDNA of HA binding peptide could express this peptide as evidenced by Western blotting.

In the next year, we will use the synthetic HA binding peptide and newly constructed expression vector to test if the *in vitro* anti-tumor effect of HA binding peptide can be translated *in vivo* against the cell growth of neurofibromatosis.

Key Research Accomplishments

In past first year, we have finished the following tasks: 1) chemical synthesis HA binding peptide and control peptide in a large scale; 2) identification of its HA binding activity; 3) characterization of anti-tumor activity of HA binding peptide; 4) study of the effect of HA binding peptide on molecules involved in cell programmed death; and 5) construction of mammalian expression vector for HA binding peptide.

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Conclusions

- HA binding peptide can be chemically synthesized in a large scale.
- The synthetic HA binding peptide possesses its bioactivity of binding to HA.
- HA binding peptide can significantly change the morphology of treated ST88-14 NF1 cells and inhibit the proliferation of ST88-14 cells in a dose-dependent manner in an anchorage-dependent condition.
- HA binding peptide binds to Bcl-2/Bcl-x_L, which may be one of the mechanisms by which HA binding peptide inhibits ST88-14 NF1 cells.
- The cells transfected with constructed vector can express HA binding peptide.

Reportable outcomes

(Due to or partially due to this support)

Papers and abstracts

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